Anaerobic treatment of crude glycerol from biodiesel production

ABSTRACT
In this study, we evaluated the use of an up-flow anaerobic sludge blanket (UASB) reactor to treat crude glycerol obtained from cottonseed biodiesel production. The laboratory-scale UASB reactor (7.0 L) was operated at ambient temperature of 26.5 °C with chemical oxygen demand (COD) concentrations between 0.5 and 8.0 g/L. The volatile fatty acid contents, pH, inorganic salt contents and biogas production were monitored during a 280-day experimental period. Molecular biology techniques were used to assess the microbial diversity in the bioreactor. The reactor achieved COD removal efficiencies of up to 92% except during one phase when the efficiency decreased to 81%. Biogas production remained stable throughout the experimental period, when the fraction converted to methane reached values as high as 68%. The profile of the denaturing gradient gel electrophoresis (DGGE) bands suggested slight changes in the microbial community during reactor operation. The overall results indicated that the crude glycerol from biodiesel production can serve as a suitable substrate for anaerobic degradation with a stable reactor performance and biogas production as long as the applied organic loads are up to 8.06 kg COD/m³·d.

Key words | cottonseed oil, DGGE, methane production, microbial diversity, UASB reactor

INTRODUCTION
The production of energy from non-renewable fossil fuels is not environmentally friendly. A good alternative energy source is biodiesel, especially because it is renewable; other advantages are its lower sulfur and aromatic substance contents, associated with high biodegradability. Currently, many countries around the world have explored the commercial use of biodiesel blends for vehicles, including the USA, Argentina, the European Union, Japan, India and Thailand (European Parliament 2009; Yusuf et al. 2011). In Brazil, the use of biodiesel mixed with diesel is growing due to new fuel legislation. The mandate for biodiesel use was set at 5% (B5) in 2010 and the Brazilian petroleum and energy agencies were studying the possibility of increasing the blend to 7% (B7) in the near future (Castanheira et al. 2014).

Biodiesel production generates approximately 10% (w/w) glycerol as a byproduct of the transesterification reaction. Although glycerol has many uses in pharmaceutical, chemical or food industries, its crude form has low commercial value due to impurities. These depend on the type of catalyst and oil (vegetal or animal) used for biodiesel production (Viana et al. 2012; Yang et al. 2012). Due to the exponential growth of biodiesel demand and production, the use of glycerol has become an important topic, once the amount generated can extrapolate the market demand.

Aiming to provide an attractive alternative for glycerol use, anaerobic digestion has been investigated as technology for aggregating value to this waste through energy production (methane and hydrogen). In most of the recent research, glycerol is reported as being co-digested with sewage sludge to improve methane production in anaerobic systems (Nghiem et al. 2014; Athanasoulia et al. 2014). Co-digestion with microalgal biomass was also recently reported (Santos-Ballardo et al. 2015).

In a few cases, glycerol is reported as the only carbon source for methane production. When crude glycerol is the only carbon source, the organic loading rate application is usually limited by salt accumulation in anaerobic systems. Methanol and calcium, magnesium and phosphorus salts are the main inhibitory compounds found in crude glycerol.
Another important aspect regards the choice of reactor configuration. Vlassis et al. (2013), using analytical glycerol in a periodic anaerobic baffle reactor (PABR), achieved good chemical oxygen demand (COD) removal efficiency by applying an organic loading rate (OLR) up to 3 kg COD/m³·d, which was much higher than the maximum of 0.25 kg COD/m·d when it was applied in a continuous stirred tank reactor (CSTR); and by using a conventional up-flow anaerobic sludge blanket (UASB) reactor operated for long periods, OLR up to 4.3 kg COD/m³·d could be applied (Hutnan et al. 2013).

Thus, considering that cottonseed is the third most important and also the cheapest raw material for biodiesel production in Brazil, crude glycerol from its production was considered as the main substrate to be used in the present work. The goal of this study was to evaluate the crude glycerol application as a direct substrate in a UASB reactor. Molecular biology techniques were also used to assess the microbial diversity changes during the bioreactor operational phases.

**METHODS**

**Glycerol characterization**

The crude glycerol used in the study was obtained from a pilot-scale plant that produced biodiesel using the transesterification process of cottonseed oil with sodium hydroxide as catalyst. The average values of crude glycerol characterization were: 1,636 g/L COD, 448.3 mg/L total organic carbon (TOC), pH 9.7, density 1,050 kg/m³, 31.6 g/L sodium, 2.0 g/L potassium, 2.9 g/L magnesium and 6.5 g/L calcium.

**UASB operation**

A laboratory-scale UASB reactor was operated at room temperature resulting in 26.5 ± 1.5 °C measured in the effluent reactor during the six phases of the experimental period (280 days). The reactor was made of acrylic (diameter 95 mm and height 1.0 m) and had a total working volume of 7.0 L (Figure 1). The inflow was set at 0.365 L/h and controlled by a peristaltic pump, resulting in average values of hydraulic retention time (HRT) of 19.2 h and up flow velocities of 0.053 m/h. The inoculum consisted of 2.5 L of flocculent sludge (94.4 g VSS/L) (VSS: volatile suspended solids) from a full-scale UASB reactor treating domestic wastewater, resulting in 33.7 g VSS/L in the laboratory reactor. The maximum specific methanogenic activity was 0.180 g COD-CH₄/g VSS·d. Crude glycerol was diluted in the influent solution to provide COD concentrations of 0.5 to 8.0 g/L (corresponding applied volumetric organic loading rates (OLR) of 0.50 to 8.06 kg COD/m³·d, respectively). This solution contained the following macronutrients (in g/L): 0.28 NH₄Cl, 0.252 K₂HPO₄, 0.1 MgSO₄·7 H₂O, 0.007 CaCl₂, 0.1 yeast extract; and 1 mL/L of micronutrient solution. This micronutrient solution was prepared as follows (in mg/L): 2.0 FeCl₂·4H₂O, 0.05 ZnCl₂, 0.5 MnCl₂·4H₂O, 0.142 NiCl₂·6H₂O, 0.05 H₃BO₃, 0.038 CuCl₂·2H₂O, 2.0 CoCl₂·6H₂O, 0.09 AlCl₃·6H₂O, 0.05 (NH₄)₆·Mo₇O₂₄·4H₂O, 1.0 EDTA and 0.2 resazurin (Florencio et al. 1995).

**Analyses**

Analytical determinations were conducted according to the APHA (2005). The COD removal efficiency was based on the raw COD in the influent and the soluble COD (filtered 1.2 μm fiberglass membrane) in the effluent. Volatile fatty acids (VFAs) were quantified using an Agilent 7,890A gas chromatograph coupled with a Thermo Scientific™ TRACE™ TR-WAX column (30 m × 0.25 mm × 0.25 μm) with a flame ionization detector (FID). The analytical conditions used in the analyses included the following: hydrogen as the carrier gas at a flow rate of 1.34 mL/min; injection port, detector and oven temperatures of 250 C,
300 °C and 300 °C, respectively; a sample injection volume of 1 μL; and a split ratio of 10:1. The carbon, hydrogen, nitrogen and sulfur concentrations from crude glycerol were determined using a CHNS-O elemental analyzer (CE Instruments, model EA 1110). The chloride and cation concentrations (sodium, potassium, magnesium, calcium and ammonium) were determined using a Thermo Scientific™ Dionex™ ion chromatography (IC) system (models ICS-2100 and ICS-1100, respectively). Detection was performed by suppressing the conductivity detector. Biogas measurements were performed using a Ritter Miligasmeters, model MGC-1 V3.1 PMMA (Germany). To control the gas pressure in the reactor headspace and to remove carbon dioxide from the biogas, a water seal containing a 10% (w/w) sodium hydroxide solution was installed before the gas meter.

Molecular biology analyses

The samples for 16S rDNA analyses were taken from the inoculum and at days 106, 138, 155, 211 and 240 of the experiment. The total genomic DNA of the samples was acquired from 0.5 g of sludge that was previously centrifuged using the commercial PowerSoil™ DNA Isolation Kit (Mobio Laboratories, California, USA). Polymerase chain reaction (PCR) amplification was performed using DNA denaturation at 94 °C for 4 min, followed by 34 cycles at 94 °C for 30 s, annealing at 48 °C for 30 s (Bacteria) or 55 °C for 30 s (Archaea), extension at 68 °C for 1 min and a final extension at 68 °C for 5 min. The Bacteria domain universal primers were 968-F (5′-AACGCGAGAAGACCTTAC-3′) and 1392-R (5′-ACGGGCGGTGTGTAC-3′); GC-clamp: CGCCCGGGGCGGGGGCGGGGGCACGGGGGGG (Nielsen et al. 1999). The Archaea domain universal primers were 1100-F (5′-AGTCAGGTAACGAGCGAG-3′) and 1400-R (5′-GTGCAAGGACGAGAAC-3′) primers; GC-clamp: CGCCCGGGGCGGGGGCGGGGGCGGGGCGGGGGGCGA-CGGGG GG (Kudo et al. 1997). The PCR products were separated by denaturing gradient gel electrophoresis (DGGE) according to Muyzer et al. (1993). Briefly, PCR products were resolved on 8% (w/v) polyacrylamide gels in a 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris, pH of 8.0, 20 mM acetic acid, 1 mM EDTA) using denaturing gradients formamide-urea ranging from 45% to 65%. Electrophoresis was performed at 220 V for 4.5 h in a D-Code (Bio-Rad Laboratories, California, USA). The electrophoresis buffer (1x TAE) was maintained at 60 °C. The gels were stained with ethidium bromide and visualized on a UV transilluminator.

RESULTS AND DISCUSSION

UASB performance

The average values of the OLR and COD removal efficiency during the six phases of the reactor operation are shown in Table 1 and Figure 2. The OLR was gradually increased from phase I (0.50 kg COD/m³·d) to phase VI (8.06 kg COD/m³·d), resulting in an average COD removal efficiency of around 85%, except for the period between phases IV and V when efficiency dropped to values of 66%. The OLR value in this phase was intentionally decreased because the VFA values increased due to the low buffering capacity of the medium in the previous stage IV (total alkalinity below 1,000 mg/L). Nevertheless, the reactor recovered its performance in phase VI despite the fact that the applied OLR of 8.06 kg COD/m³·d had reached the maximum value of the entire experimental period; and it was also when the highest COD removal efficiency of 92% was obtained.

Regarding the influence of salts in the crude glycerol containing influent, increasing the OLR resulted in a naturally increased influent chloride, sodium and conductivity values, with maximum values of 500 mg/L, 727 mg/L, and 3,027 μS/cm, respectively. Sodium concentrations of 5, 10, and 14 g Na+/L caused inhibition of 10, 50, and 100% in

![Table 1](https://iwaponline.com/wst/article-pdf/72/8/1383/466257/wst072081383.pdf)
the methanogenic acetoclastic activity, respectively (Rinzema et al. 1988). Sodium and potassium would strongly inhibit microbial growth during glycerol anaerobic degradation at concentrations of 8 g Na+/L to or 12 g K+/L (Viana et al. 2012). Hutnan et al. (2013) operated a UASB reactor fed with crude glycerol and reported a sudden failure after 440 days of operation, which was attributed to the dissolved inorganic salts content, which reached 30 g/L. In this study, it is unlikely that inhibition by inorganic salts occurred because the glycerol dilution in the influent was high (on average 1:2500 in phase I up to 1:530 in phase VI). The fact that methane production remained stable during the experimental period confirms the non-inhibitory effect of those salts.

The COD removal efficiency measured as methane ranged from 64.7 to 68.0%, except in phases IV and V, when these values decreased to 53.8% and 54.6%. The total COD removal efficiency was maintained above 80% in all the phases with the lowest values also occurring during phases IV and V. During phase I, the goal was to adapt the biomass to crude glycerol. That is why the average COD removal efficiency was 86.1% when the applied OLR was only 0.50 kg COD/m³·d. After the sludge adaptation in phase I, the COD removal efficiencies reached 92.5% in phases II and III when the OLR was increased to 2.65 kg COD/m³·d.

However, when the OLR was increased 5.72 kg COD/m³·d in phase IV, the reactor operation became unstable, which resulted in foam formation and sludge washout. The result was a COD removal efficiency decrease to a minimum value of 66.4%. The effluent VFA and COD concentrations increased to 420 mg HAc/L and 520 mg O2/L, respectively. Nevertheless, a nearly neutral pH could be maintained. To avoid further operational problems, the OLR was reduced to 4.09 kg COD/m³·d in phase V; additionally, 4.0 g/L NaHCO₃ was added to the reactor to enhance the buffer capacity. Finally, after 205 days of operation, the OLR was increased to 8.06 kg COD/m³·d in phase VI, as planned initially for the reactor operation. The result was an extremely good COD removal efficiency of 92%.

Comparing the COD removal and applied OLR during the experimental period with those of previous similar works, the results in this research can be considered a high achievement, especially because of the use of crude glycerol. Vlassis et al. (2012) studied the anaerobic digestion using a CSTR but fed with pure glycerol; however, when the applied OLR was above only 0.25 kg COD/m³·d, reactor performance was poor due to VFA accumulation and pH decrease. The authors achieved maximum biogas and methane production of 0.42 ± 0.05 L/g COD and 0.30 ± 0.04 L/g COD, respectively. Yang et al. (2008) used a fixed-bed bioreactor packed with polyurethane foam in semi-continuous mode, but fed with synthetic glycerol containing wastewater. They obtained good biodegradation efficiency under mesophilic and thermophilic conditions. The methane yield decreased when the OLR increased under mesophilic conditions. Conversely, there was a relatively higher methane yield under thermophilic conditions. Nevertheless, the maximum OLR studied was only 1.00 kg COD/m³·d.

In other works, good COD removal efficiencies and higher OLR could be applied, but after some previous pretreatment. Nuchdang & Phalakornkule (2012) obtained total COD removal efficiency of 86% and methane content of 54% with a UASB reactor fed with crude glycerol from waste cooking oil, but after pretreatment and acidification.
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**Figure 3** | DGGE gel from Bacteria domain during the UASB experiment. I: inoculum; T (top), M (middle) and B (bottom) collection points at heights of 43 cm, 26 cm and 12 cm from the bottom of the reactor, respectively.

**Figure 4** | DGGE gel from Archaea domain during the UASB experiment. I: inoculum; T (top), M (middle) and B (bottom) collection points at heights of 43 cm, 26 cm and 12 cm from the bottom of the reactor, respectively.
The applied OLR values were between 1.3 and 2.6 kg COD/m³·d. Hüttan et al. (2015) observed a good COD removal efficiency and stable biogas production with high methane content, when operating a UASB with OLR up to 12 kg COD/m³·d during long periods. This high value can be explained by the use of glycerol pretreated by acidification, which resulted in a higher influent VFA content (80%).

These previous studies corroborate the findings of the present work, demonstrating the feasibility of anaerobic digestion of even crude glycerol, with good COD removal efficiency at high applied OLR, to boost methane production. However, the feed should be carefully controlled in terms of influent alkalinity and organic loadings compatible with the sludge activity and adaption, in order to prevent adverse conditions.

**Microbial diversity**

The band profiles of the sludge related to the Bacteria domain suggested slight changes in the bacterial community during the reactor operation (Figure 3). However, several common bands at different sampling points and at different days of operation, which are readily visualized, indicated the presence of a bacterium that plays an important role during the process. The presence of a smear (a ‘track’ formed by the high number of extremely faint bands on the gel) in all of the DGGE gel samples in the Archaea domain (Figure 4) could potentially result from the high diversity of species present in the anaerobic process. A new band (e) started to appear between phase III and IV when the system was disturbed. However, when the reactor was recovered, this band reduced its intensity, and may be related to microbes being more resistant to VFA changes or by-products degradation found in phase V.

**CONCLUSIONS**

Crude glycerol from biodiesel production can serve as a suitable and main substrate for anaerobic degradation, which results in stable reactor performance with good biogas production when organic loadings are applied at up to 8.06 kg COD/m³·d. The total COD removal efficiency can reach 92%, and the fraction converted to methane can be up to 68%. It is important to provide sufficient alkalinity to avoid the reduction of COD efficiency due to higher acidification during reactor high loading. Few changes occurred in the microbial communities regarding the Bacteria domain as the organic loadings changed. However, a high diversity of Archaea species was observed. Additional studies are underway to optimize methane production, as well as to elucidate the microbial communities involved in the anaerobic treatment of crude glycerol by using a pyrosequencing method.

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